REMARKS

Applicants recognize the inadvertent inclusion of the earlier cancelled claims 8 and 9, and as claim 10 is cancelled herein, recognize claims 1-2, 4-7 and 11 only, as properly under consideration in the office action.

The examiner rejects claims 1-2, 4-7 and 11 under 35 USC §112, second paragraph, and also objects to claim 4 under 37 CFR §1.75(c). Applicants submit that the above amendments bring these claims into conformance with these requirements, and respectfully request withdrawal of the rejections/objections.

Support for the amendment to claim 1 comes from Example 10 of the specification (found on page 16). This example states that the esterase designated PFE-wt, i.e., wild-type esterase from *Pseudomonas fluorescens*, shows no ability to hydrolyze a racemic mixture of the designated compound 1. The mutated esterase PFE-U3, obtained following the process of the present invention, does show such an ability to hydrolyze this compound. Applicants submit that the amending language is supported by this disclosure, and that this language indicates clearly the concept of an "altered" substrate specificity as envisioned by the presently claimed invention.

The examiner further rejects claims 1-2, 4-7 and 11 under 35 USC §102(b) as anticipated by Greener, et al. (Meth. Mol. Biol. 57:375-385). On page 7 of the office action, the examiner indicates that Greener does not teach the screening and identification of an altered substrate specificity as presently defined in claim 1 (office action, p.7). In other words, this reference discloses a method for obtaining a mutated enzyme showing an increase in an already known and detectable enzymatic activity,

but does not teach that this mutagenesis and screening process can introduce a previously undetectable enzymatic activity into an enzyme, such as the examples in the specification demonstrate with respect to the 3-hydroxyester (see p.11:1-36 and p.16:10-27). Applicants therefore respectfully request that the examiner withdraw the present rejection under 35 USC §102(b).

Finally, the examiner rejectes claims 1-2, 4-7 and 11 under 35 USC §103(a) as obvious over Greener, et al. (above) in view of Wilks, et al. (Curr. Op. Biotech., 1991, 2:561-567). Applicants wish to thank the examiner for providing the correct Wilks reference on which the rejection is based, and recognize their role in the confusion on this point. However, applicants respectfully traverse the rejection based on this Wilks reference, as well, as the cited examples do not seem to conclusively support the examiner's assertions. We are unable to find in Wilks any support for the assertion that the ordinarily skilled worker would be motivated thereby to use random mutagenesis to introduce a "new" specificity into an enzyme. Additionally, applicants find that overall, the disclosure of Wilks suggests that this methodology is not efficient or desireable when compared to the rational design method.

As indicated above, and as recognized specifically by the examiner, Greener does not disclose success in obtaining an enzyme having a 'new' substrate specificity, by random mutagenesis. The examiner then states that Wilks, however, "*clearly* teach[es] the *ease* with which 'new' specificities are generated, *either by directed or random mutagenesis*," and that "the random methods of Greener et al. would reasonably produce the same result [as Wilks] given the appropriate amount of

experimentation" (office action, p.8, emphasis supplied). Applicants suggest that this is not the case.

Two of the cited examples give a fairly strong indication that the allegedly "new" specificity existed, objectively and measureably, prior to the rational redesign, and the third example actually produces a "new" product, with no "new" substrate specificity. The first two of these involve NADH and NADPH, and the third involves 2-octanol.

In the L-lactate dehydrogenase example, a simple, one site-specific rational redesign "achieved a *20-fold shift* in the coenzyme specificity *towards* NADPH" (p.564, emphasis added). Applicants submit that this *shift* indicates a detectable base level of specificity *for* NADPH prior to the redesign. Such preexisting specificity also appears in the statement that the *shift* came "mainly as a result of the weakened binding of NADH," rather than in an increased affinity *for* NADPH (p.564). Accordingly, applicants suggest that it can reasonably be assumed that both the affinity for, and the catalysis of, NADPH continually existed in this enzyme, and that NADH is the recognized coenzyme mainly (or solely) because it binds more quickly to the native enzyme. Therefore, substrate specificity appears to have existed for NADPH, and was not, in fact "new."

The next cited example reports a mutant glutathione reductase (requiring seven site-specific mutations) showing "a *shift* in substrate specificity *18,000 fold* away from NADPH toward NADH" (p.564, emphasis added). Again, the description's use of the word *shift* indicates a detectable base level of specificity *for* NADH prior to the redesign. Applicants respectfully submit that neither of these examples shows *clear* indication

that the respective coenzyme is a "new" substrate as applicants understand and claim this in the present invention.

The third example cited by the examiner, used to reject claim 11, allegedly teaches the "engineering of a double mutant of alcohol dehydrogenase ... altered from utilizing only a single isomer of 2-octanol to using both stereoisomers" (office action p.9). Applicants read the language of Wilks differently. There, Wilks states that "[f]rom the Trp93Ala/Thr48Ser double mutant and 2-octanal, both R- and S-isomers of 2-octanol *are produced*" (p.563, emphasis added). The substrate of this mutant is 2-*octanal* prior to redesign, and it *remains* 2-octanal after redesign. The difference is in the *product*, of which difference Wilks states, "[t]hus the usefulness of this mutant for chiral synthesis is diminished" (p.563). Applicants suggest that this directly contradicts the examiner's motivation argument that "'new' substrate specificities are useful 'for the purpose of making [enzymes] more suitable for the chemoenzymatic synthesis of ... **chiral** compounds'" (office action, p.9, citing Wilks, Abstract, emphasis by the examiner).

Rather than "clearly teach[ing] the ease with which 'new' specificities are generated," applicants suggest that none of these cited examples *clearly* shows that "new" specificities were generated at all (office action, p.8). Even in the single mutant of the alcohol dehydrogenase discussed above shows "markedly *greater* activity towards aliphatic alcohols," not a newly observable activity (Wilks, p.563). To obtain a "broad substrate-specificity α-hydroxyacid dehydrogenase" from the *Bacillus stearothermophilus* lactate dehydrogenase, one group redesigned the catalytic vacuole

of this enzyme using mouse and rat testicular LDH structure (Wilks, p.563). To accomplish this redesign, the vacuole needed five amino acid substitutions on two different regions of the protein. These mutations "decreas[ed] the size and increas[ed] the hydrophobicity of the amino acids lining the catalytic vacuole" (p.563) Wilks comments on the process by saying "[u]sing this well understood framework [i.e., altering the size and hydrophobicity of the vacuole], rational engineering enables coupled changes in enzyme structure to be obtained with greater probability of success than random point mutagenesis" (p.563).

As this indicates, the success in creating an enzyme having a "new" substrate specificity, especially given the generally more complex changes potentially involved, depends mainly on the degree to which the framework of the enzyme has been studied and understood. Accordingly, even though Wilks suggests that random mutagenesis *might* be used to "construct[] proteins with enzyme *properties* that are not readily available in nature," that reference explicitly states that study of structure and rational engineering is more likely to bring success (p.561). Additionally, the word *properties*, and the single example of random mutagenesis presented, support this position that Wilks would not suggest random mutagenesis for creating "new" substrates.

"Properties" suggests more than simply the creation of an enzyme with a "new" (altered) substrate specificity as presently claimed. The examiner's definition of a "new" substrate specificity in the office action recognizes that "'a variant having higher specific activity'" can be considered "new" (p.7, citing Greener, p.384). Given that Wilks' abstract also uses the term "new" to describe all differences in substrate activity, in that

reference we find it reasonable to imply that the "new" "enzyme *properties*" include increases in stability or activity on a particular substrate.

As has been indicated above, and as can be readily ascertained from the examples, Wilks does, indeed, suggest this interpretation of both "new" and "enzyme properties." Given this point, the disclosure of a specific example of random mutagenesis, and the above statement of preference for rational design, applicants submit that random mutagenesis would be seen by the skilled artisan as unprofitable for the purposes of the presently claimed invention.

The only example of random mutagenesis produced an enzyme with six amino acid mutations, all of which were structural in relation to the active site, and which together served to *stabilize* the enzyme and increase its esterase activity in relation to its protease activity (first serine protease example, p.561-2). This random mutation produced an enzyme with no changes to its active site, and the only fairly clear example of a "new" specificity (as defined in the present claims) required a redesign of the catalytic vacuole, requiring five mutations to a specific portion of the enzyme. Coupled with the statement that understanding the structure and rationally engineering the protein gives a "greater probability of success" for coupled changes than random mutagenesis, these points indicate a strong suggestion that random mutagenesis is very much of lower potential for obtaining "new" substrate specificities according to the present invention.

The examiner states that an enablement rejection based on the unpredictability of random mutagenesis "in producing ... a 'new' enzyme" was withdrawn, as "Greener

et al. ... accomplished such a feat using the same methods as applicants" (office action p.15). The methodology employed by Greener did not, in fact, accomplish the same feat as claimed by the applicants. In Greener, the random mutagenesis process produced a phosphatase with increased specific activity, and not a phosphatase with a previously undetectable activity. The production of this phosphatase mimics the production of the mutant serine protease in Wilks. Neither reference suggests that a "new," previously undetected, activity might result from the random mutagenesis of an enzyme gene, even "given the appropriate amount of experimentation" (office action, p.8).

Applicants respectfully submit that the only example demonstrating that an enzyme with a previously undetected specificity can be obtained by random mutagenesis is shown in applicants specification. The limitation of Greener's research to optimization of a known specificity, and Wilks' likewise limited exploration of random mutagenesis, coupled with Wilks' statement that a broadening of specificity is best accomplished through a "well understood framework [and] rational engineering," suggests clearly to applicants that production of "new" specificities by the presently claimed process went against the then-extant understanding of the art.

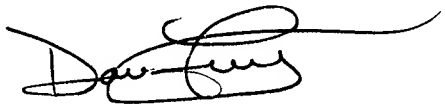
Accordingly, the combination of the references cited would lead one of ordinary skill in the art away from the presently claimed process. Any understanding of a possibility for random mutagenesis to produce significant changes in specificity as envisioned by applicants would be accompanied by an understanding that such a change would likely take a great deal of work. It might be possible, but there seems to

have been little hope for true success. The presently claimed invention is successfully demonstrated in the present specification. We respectfully request that the examiner withdraw the rejection of claims 1-2, 4-7 and 11 under 35 USC §103(a).

In view of the foregoing amendments and remarks, applicants consider that the rejections of record have been obviated and respectfully solicit passage of the application to issue.

Please charge any shortage in fees due in connection with the filing of this paper, including Extension of Time fees to Deposit Account No. 11-0345. Please credit any excess fees to such deposit account.

Respectfully submitted,
KEIL & WEINKAUF



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VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE CLAIMS

Please cancel claim 10.

Please amend claims 1, 4 and 11 to read as follows:

1. A method for altering the substrate specificity of an enzyme such that its specificity relative to a substrate on which no specificity can initially be detected increases to a level where specificity on that substrate is detected, which method comprises the steps of:

a) introducing a DNA which comprises a copy of the gene coding for the enzyme into the Escherichia coli strain XL1 Red or into a functional derivative,

b) incubating the transformed Escherichia coli strain XL1 Red or its functional derivative to generate mutations in the enzyme gene,

c) transferring the mutated DNA from the strain XL1 Red or its functional derivative to a microorganism which has no impeding enzyme activity,

d) incubating this microorganism to detect the enzyme activity in at least one selection medium which comprises at least one enzyme substrate to recognize altered substrate specificity of the enzyme, with or without other indicator substances,

e) selecting the microorganisms which show an alteration in the substrate specificity, said microorganisms in steps b), d) and e) being a member selected from the group consisting of bacteria, fungi and yeasts.

4. A method as claimed in claim 1, wherein the microorganisms [bacteria] are selected from the group consisting of Gram-positive and Gram negative bacteria.

11. A method as claimed in claim 1 [10], wherein the alteration in the substrate specificity results in a stereoselective enzyme activity.